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(21) International Application Number: PCT/US88/01811 (22) International Filing Date: 26 May 1988 (26.05.88) (31) Priority Application Numbers: 054,369 168,190 (32) Priority Dates: 26 May 1987 (26.05.87) 15 March 1988 (15.03.88) (33) Priority Country: US (71) Applicant: CALGENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US). (72) Inventors: HOUCK, Catherine, M. ; 172 Carmel Court, Vacaville, CA 95688 (US). PEAR, Julie, R. ; 710 Arthur Street, Davis, CA 95616 (US). (74) Agent: RAE-VENTER, Barbara; Leydig, Voit & Mayer, 350 Cambridge Avenue, Suite 200, Palo Alto, CA 94306 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: FRUIT-SPECIFIC TRANSCRIPTIONAL FACTORS (57) Abstract Fruit-specific regulatory regions are identified employing cDNA screening. The resulting fruit-specific regulatory regions are manipulated for use with foreign sequences for introduction into plant cells to provide transformed plants having fruit with a modified phenotypic property. The invention is exemplified with a tomato fruit-specific promoter which is active throughout the stages of fruit ripening.		

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FRUIT-SPECIFIC
TRANSCRIPTIONAL FACTORS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Serial No. 168,190, filed March 15, 1988, which is a continuation-in-part of Application Serial
10 No. 054,369 filed May 26, 1987, which applications are incorporated herein by reference.

INTRODUCTION

Technical Field

15 This invention relates to DNA expression cassettes capable of directing fruit-specific expression of in vitro constructed expression cassettes in plants. The invention is exemplified by promoters useful in fruit-specific transcription in a tomato plant.

20 Background

Manipulation of plants has proven to be significantly more difficult than manipulation of prokaryotes and mammalian hosts. As compared to
25 prokaryotes and mammalian cells, much less was known about the biochemistry and cell biology of plant cells and plants. The ability to transform plant cells and regenerate plants is unique to flora since other differentiated species provide readily available trans-
30 formable germ cells which may be fertilized and introduced into the live host for fetal development to a mature fetus. There has been substantial interest in modifying the ovum with inducible transcriptional initiation regions to afford inducible transcription and
35 expression of the gene introduced into the ovum, rather than constitutive expression which would result in expression throughout the fetus.

Also, for plants, it is frequently desirable to be able to control expression at a particular stage in the growth of the plant or in a particular plant part. During the various stages of the growth of the plant, and as to the various components of the plant, it will frequently be desirable to direct the effect of the construct introduced into the entire plant or a particular part and/or to a particular stage of differentiation of the plant cell. For this purpose, regulatory sequences are required which afford the desired initiation of transcription in the appropriate cell types and/or at the appropriate time in the plant development, without having serious detrimental effects on the plant development and productivity.

It is therefore important to be able to isolate sequences which can be manipulated to provide the desired regulation of transcription in a plant cell host during the growing cycle of the plant. One aspect of this interest is the ability to change the phenotype of fruit, so as to provide fruit which will have improved aspects for storage, handling, cooking, organoleptic properties, freezing, nutritional value, and the like.

25 Relevant Literature

cdNA clones from tomato displaying differential expression during fruit development have been isolated and characterized (Mansson et al., Mol. Gen. Genet. (1985) 200:356-361; Slater et al., Plant Mol. Biol. (1985) 5:137-147). The studies have focused primarily on mRNAs which accumulate during fruit ripening. One of the proteins encoded by the ripening-specific cDNAs has been identified as polygalacturonase (Slater et al., Plant Mol. Biol. (1985) 5:137-147). A cdNA clone which encodes tomato polygalacturonase has been sequenced. Grierson et al., Nucleic Acids Research (1986) 14:8395-8603. The concentration of polygalac-

turonase mRNA increases 2000-fold between the immature-green and red-ripe stages of fruit development. This suggests that expression of the enzyme is regulated by the specific mRNA concentration which in turn is regulated by an increase in transcription. Della Penna et al., Proc. Natl. Acad. Sci. USA (1986) 83:6420-6424.

Mature plastid mRNA for psbA (one of the components of photosystem II) reaches its highest level late in fruit development, whereas after the onset of ripening, plastid mRNAs for other components of photosystem I and II decline to nondetectable levels in chromoplasts.

Piechulla et al., Plant Mol. Biol. (1986) 7:367-376.

Other studies have focused on cDNAs encoding genes under inducible regulation, e.g. proteinase inhibitors which are expressed in response to wounding in tomato (Graham et al., J. Biol. Chem. (1985) 260:6555-6560; Graham et al., J. Biol. Chem. (1985) 260:6561-6564) and on mRNAs correlated with ethylene synthesis in ripening fruit and leaves after wounding. Smith et al., Planta (1986) 168:94-100.

Leaf disc transformation of cultivated tomato is described by McCormick, et al., Plant Cell Reports (1986) 5:81-89.

SUMMARY OF THE INVENTION

Novel DNA constructions are provided employing a "fruit-specific promoter," particularly those active beginning at or shortly after anthesis or beginning at the breaker stage, joined to a DNA sequence of interest and a transcriptional termination region. A DNA construct may be introduced into a plant cell host for integration into the genome and transcription regulated at a time at or subsequent to anthesis. In this manner, high levels of RNA and, as appropriate, polypeptides, may be achieved during formation and/or ripening of fruit.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of the cDNA clones pCGN1299 (2A11) and pCGN1298 (3H11). The amino acid sequence of the polypeptide encoded by the open reading frame is also indicated.

Figure 2 is a comparison of 2A11 to pea storage proteins and other abundant storage proteins:

(a) 2A11 (residues 33-46) is compared to PA1b and the reactive site sequences of some protease inhibitors, PA1b (residues 6-23), chick pea inhibitor (residues 11-23), lima bean inhibitor (residues 23-35), human α 1-antitrypsin reactive site peptide. The arrow indicates the reactive site.

(b) is a comparison of the amino terminal sequence of 2A11 with the amino termini of a range of seed proteins. The data have been modified or deletions introduced to maximize homology; conserved residues are shown boxed. The sequences are from the following sources: PA1b; barley chloroform/methanol-soluble protein d; wheat albumin; wheat α -amylase inhibitor 0.28; millet bi-functional inhibitor; castor bean 2S small subunit; and napin small subunit.

Figure 3 is a schematic diagram of the construction of the binary plasmid pCGN783; (a) through (f) refer to the plasmid constructions in Example 6.1.

Figure 4 shows the complete sequence of the 2A11 genomic DNA cloned into pCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654).

Figure 5 shows the nucleotide sequence of a polygalacturonase (PG) genomic clone.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, DNA constructs are provided which allow for modification of plant phenotype during fruit maturation and ripening. The DNA constructs provide for a regulated transcrip-

tional initiation region associated with fruit development and ripening. Downstream from and under the transcriptional initiation regulation of the fruit related initiation region will be a sequence of interest which will provide for modification of the phenotype of the fruit. Desirably, integration constructs may be prepared which allow for integration of the transcriptional cassette into the genome of a plant host. Conveniently, the vector may include a multiple cloning site downstream from the fruit related transcriptional initiation region, so that the integration construct may be employed for a variety of sequences in an efficient manner.

Of particular interest is a transcriptional initiation region which is activated at or shortly after anthesis, so that in the early development of the fruit, it provides the desired level of transcription of the sequence of interest. Normally, the sequence of interest will be involved in affecting the process in the early formation of the fruit or providing a property which is desirable during the growing (expansion) period of the fruit, or at or after harvesting.

The ripening stages of the tomato may be broken down into mature green, breaker, turning, pink, light red and red. Desirably, the transcriptional initiation region maintains its activity during the expansion and maturation of the green fruit, more desirably continues active through the ripening or red fruit period. Comparable periods for other fruit are referred to as stages of ripening. The invention is not limited to those transcriptional initiation regions which are activated at or shortly after anthesis but also includes transcriptional initiation regions which are activated at any of the ripening stages of the fruit.

The transcriptional initiation region may be native or homologous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. Of particular interest is a tomato fruit-specific transcriptional initiation region referred to as 2A11 which regulates the expression of a 2A11 cDNA sequence described in the Experimental section. The 2A11 transcriptional initiation region provides for an abundant messenger, being activated at or shortly after anthesis and remaining active until the red fruit stage. The expressed protein is a sulfur-rich protein similar to other plant storage proteins in sulfur content and size. Also of interest is the transcriptional initiation region which regulates expression of the enzyme polygalacturonase, an enzyme which plays an important role in fruit ripening. The polygalacturonase promoter is active in at least the breaker through red fruit stage.

Other fruit-specific promoters may be activated at times subsequent to anthesis, such as prior to or during the green fruit stage, during pre-ripe (e.g., breaker) or even into the red fruit stage.

A transcriptional initiation region may be employed for varying the phenotype of the fruit. Various changes in phenotype are of interest. These changes may include up- or down-regulation of formation of a particular saccharide, involving mono- or polysaccharides, involving such enzymes as polygalacturonase, levansucrase, dextranase, invertase, etc.; enhanced lycopene biosynthesis; cytokinin and monellin synthesis. Other properties of interest for modification include response to stress, organisms, herbicides, bruising, mechanical agitation, etc., change in growth regulators, organoleptic properties, etc. For antisense or complementary sequence transcription, the sequence will

usually be at least 12, more usually at least 16 nt. Antisense sequences of interest include those of polygalacturonase, sucrase synthase and invertase.

5 The transcriptional cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a sequence of interest, and a transcriptional and translational termination region functional in plants. One or more introns may be also be present. The DNA sequence may
10 have any open reading frame encoding a peptide of interest, e.g. an enzyme, or a sequence complementary to a genomic sequence, where the genomic sequence may be an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, e.g. splicing, or translation.
15 The DNA sequence of interest may be synthetic, naturally derived, or combinations thereof. Depending upon the nature of the DNA sequence of interest, it may be desirable to synthesize the sequence with plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest.

25 In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed for
30 joining the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. Toward this end, in vitro mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be employed, where
35 insertions, deletions or substitutions, e.g. transitions and transversions, may be involved.

The termination region which is employed will be primarily one of convenience, since the termination regions appear to be relatively interchangeable. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions.

By appropriate manipulations, such as restriction, chewing back or filling in overhangs to provide blunt ends, ligation of linkers, or the like, complementary ends of the fragments can be provided for joining and ligation.

In carrying out the various steps, cloning is employed, so as to amplify the amount of DNA and to allow for analyzing the DNA to ensure that the operations have occurred in proper manner. A wide variety of cloning vectors are available, where the cloning vector includes a replication system functional in E. coli and a marker which allows for selection of the transformed cells. Illustrative vectors include pBR332, pUC series, M13mp series, pACYC184, etc. Thus, the sequence may be inserted into the vector at an appropriate restriction site(s), the resulting plasmid used to transform the E. coli host, the E. coli grown in an appropriate nutrient medium and the cells harvested and lysed and the plasmid recovered. Analysis may involve sequence analysis, restriction analysis, electrophoresis, or the like. After each manipulation the DNA sequence to be used in the final construct may be restricted and joined to the next sequence, where each of the partial constructs may be cloned in the same or different plasmids.

In addition to the transcription construct, depending upon the manner of introduction of the transcription construct into the plant, other DNA sequences may be required. For example, when using the Ti- or Ri-plasmid for transformation of plant cells, as described below, at least the right border and frequently both the right and left borders of the T-DNA of the Ti- or Ri-plasmids will be joined as flanking regions to the transcription construct. The use of T-DNA for transformation of plant cells has received extensive study and is amply described in EPA Serial No. 120,516, Hoekema, In: The Binary Plant Vector System Offset-drukkerij Kanters B.V., Alblasterdam, 1985, Chapter V, Knauf et al., Genetic Analysis of Host Range Expression by Agrobacterium, In: Molecular Genetics of the Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, NY, 1983, p.245, and An et al., EMBO J. (1985) 4:277-284

Alternatively, to enhance integration into the plant genome, terminal repeats of transposons may be used as borders in conjunction with a transposase. In this situation, expression of the transposase should be inducible, so that once the transcription construct is integrated into the genome, it should be relatively stably integrated and avoid hopping.

The transcription construct will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide, particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced.

A variety of techniques are available for the introduction of DNA into a plant cell host. These techniques include transformation with Ti-DNA employing A. tumefaciens or A. rhizogenes as the transforming agent, protoplast fusion, injection, electroporation,

etc. For transformation with Agrobacterium, plasmids can be prepared in E. coli which plasmids contain DNA homologous with the Ti-plasmid, particularly T-DNA.

The plasmid may or may not be capable of replication in Agrobacterium, that is, it may or may not have a broad spectrum prokaryotic replication system, e.g., RK290, depending in part upon whether the transcription construct is to be integrated into the Ti-plasmid or be retained on an independent plasmid. By means of a helper plasmid, the transcription construct may be transferred to the A. tumefaciens and the resulting transformed organism used for transforming plant cells.

Conveniently, explants may be cultivated with the A. tumefaciens or A. rhizogenes to allow for transfer of the transcription construct to the plant cells, the plant cells dispersed in an appropriate selective medium for selection, grown to callus, shoots grown and plantlets regenerated from the callus by growing in rooting medium. The Agrobacterium host will contain a plasmid having the vir genes necessary for transfer of the T-DNA to the plant cells and may or may not have T-DNA. For injection and electroporation, disarmed Ti-plasmids (lacking the tumor genes, particularly the T-DNA region) may be introduced into the plant cell.

As a host cell, any of a number of fruit bearing plants may be employed in which the plant parts of interest are derived from the ovary wall. These include true berries such as tomato, grape, blueberry, cranberry, currant, and eggplant; stone fruits (drupes) such as cherry, plum, apricot, peach, nectarine and avocado; compound fruits (druplets) such as raspberry and blackberry. In hesperidium (oranges, citrus), the expression cassette might be expected to be expressed in the "juicy" portion of the fruit. In pepos (such as watermelon, cantelope, honeydew, cucumber and squash) the equivalent tissue for expression is most likely the inner edible portions, whereas in legumes (such as

peas, green beans, soybeans) the equivalent tissue is the seed pod.

Identifying useful transcriptional initiation regions may be achieved in a number of ways. Where a fruit protein has been or is isolated, it may be partially sequenced, so that a probe may be designed for identifying messenger RNA specific for fruit. To further enhance the concentration of the messenger RNA specifically associated with fruit, cDNA may be prepared and the cDNA subtracted with messenger RNA or cDNA from non-fruit associated cells. The residual cDNA may then be used for probing the genome for complementary sequences, using an appropriate library prepared from plant cells. Sequences which hybridize to the cDNA may then be isolated, manipulated, and the 5'-untranslated region associated with the coding region isolated and used in expression constructs to identify the transcriptional activity of the 5'-untranslated region. In some instances, a probe may be employed directly for screening a genomic library and identifying sequences which hybridize to the probe. The sequences will be manipulated as described above to identify the 5'-untranslated region.

As an example, a promoter of particular interest for the subject invention, the fruit-specific transcriptional initiation region (promoter) from a DNA sequence which encodes a protein described as 2A11 in the Experimental section was identified as follows. cDNA clones made from ripe fruit were screened using cDNA probes made from ripe fruit, green fruit, and leaf mRNA. Clones were selected having more intense hybridization with the fruit DNAs as contrasted with the leaf cDNAs. The screening was repeated to identify a particular cDNA referred to as 2A11. The 2A11 cDNA was then used for screening RNA from root, stem, leaf, and seven stages of fruit development after the mRNA was sized on gels. The screening demonstrated that the

particular message was present throughout the seven stages of fruit development. The mRNA complementary to the specific cDNA was absent in other tissues which were tested. The cDNA was then used for screening a genomic library and a fragment selected which hybridized to the subject cDNA. The 5' and 3' non-coding regions were isolated and manipulated for insertion of a foreign sequence to be transcribed under the regulation of the 2A11 promoter.

10 The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al., Plant Cell Reports (1986) 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or
15 different strains, identifying the resulting hybrid having the desired phenotypic characteristic. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested for use to provide
20 fruits with the new phenotypic property.

 A protein is provided having the sequence described in the Experimental section designated as 2A11. This protein could be a storage protein and be useful in enhancing sulfur containing amino acids (cysteine
25 and methionine) in the diet. It can be obtained in substantially pure form by providing for expression in prokaryotes or eukaryotes, e.g., yeast by inserting the open reading frame into an expression cassette containing a transcriptional initiation region. A variety of
30 expression cassettes are commercially available or have been described in the literature. See, for example, U.S. Patent Nos. 4,532,207; 4,546,082; 4,551,433; and 4,559,302. The product, if intracellular, may be isolated by lysing of the cells and purification of the
35 protein using electrophoresis, affinity chromatography, HPLC extraction, or the like. The product may be isolated in substantially pure form free of other plant

products, generally having at least about 95% purity, usually at least about 99% purity.

The following examples are offered by way of illustration and not by limitation.

5

EXPERIMENTAL

Example 1

Construction of Tomato Ripe Fruit cDNA Bank and Screening for Fruit-Specific Clones

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Tomato plants (Lycopersicon esculentum cv UC82B) were grown under greenhouse conditions. Poly(A)⁺RNA was isolated as described by Mansson et al., Mol. Gen. Genet. (1985) 200:356-361. The synthesis of cDNA from poly(A)⁺ RNA prepared from ripe fruit, cloning into the PstI site of the plasmid pUC9 and transformation into an E. coli vector were all as described in Mansson et al., Mol. Gen. Genet. (1985) 200:356-361.

15

Library Screening

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Two thousand recombinant clones were screened by colony hybridization with radiolabeled cDNA made from tomato red fruit mRNA, immature green fruit mRNA, and leaf mRNA. Bacterial colonies immobilized onto GeneScreen Plus filters (New England Nuclear), were de-natured in 1.5 M NaCl in 0.5 M NaOH, then neutralized in 1.5 M NaCl in 0.5 M Tris-HCl pH 8, and allowed to air dry. Hybridization, washing and autoradiography were all performed as described in Maniatis et al., Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor, New York.

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Sixty-five clones were selected which had more intense hybridization signals with fruit cDNA than with leaf cDNA and therefore appeared to be under-represented in the leaf mRNA population relative to the fruit population. Replicate slot blot filters were prepared using purified DNA from the selected clones and hybrid-

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ized with radioactive cDNA from leaf, green fruit, and red fruit as before. This allowed selection of cDNA clone 2A11, also referred to as pCGN1299 which is on at high levels in both the fruit stages (red and green) and off in the leaf.

Example 2

Analysis of Clones

Synthesis of RNA Probes

The cDNA insert of pCGN1299 was excised as an EcoRI to HindIII fragment of approximately 600 bp (as measured on an agarose gel), and subcloned into the Riboprobe vector pGEM1 (Promega Biotec), creating pCGN488. ³²P-labeled transcripts made from each strand of the pCGN488 insert using either SP6 or T7 polymerase were used as probes in separate Northern blots containing mRNA from leaf, immature green and mature red fruits. The RNA transcript from the SP6 promoter did not hybridize to the tomato mRNA. However, the transcript from the T7 promoter hybridized to an mRNA of approximately 700 nt in length from the green fruit and the red fruit but not to mRNA from tomato leaf. The direction of transcription of the corresponding mRNA was thus determined.

The tissue specificity of the pCGN1299 cDNA was demonstrated as follows. RNA from root, stem, leaf, and seven stages of fruit development (immature green, mature green, breaker, turning, pink, light red, and red) was sized on formaldehyde/agarose gels according to the method described by Maniatis et al., (1982), immobilized on nitrocellulose and hybridized to ³²P-labeled RNA which was synthesized in vitro from pCGN488 using T7 polymerase. Each lane contained 100 ng of polyA⁺ RNA except for two lanes (pink and light red lanes) which contained 10 µg of total RNA. The Northern analysis of mRNA from root, stem, leaf, and various stages of fruit development indicated that

pCGN1299 cDNA was expressed in all stages of fruit development from the early stages immediately after anthesis to red ripe fruit. No mRNA hybridizing to pCGN1299 was found in leaf, stem, or root tissue. The size of the mRNA species hybridizing to the pCGN488 probe was approximately 700 nt.

Message abundance corresponding to the pCGN1299 cDNA was determined by comparing the hybridization intensity of a known amount of RNA synthesized in vitro from pCGN488 using SP6 polymerase to mRNA from red tomato fruit in a Northern blot. The ³²P-labeled transcript from pCGN488 synthesized in vitro using T7 polymerase was used as a probe. The Northern analysis was compared to standards which indicated that the pCGN1299 cDNA represents an abundant mRNA class in tomato fruit, being approximately 1% of the message.

Example 3

Sequencing of pCGN1299 and pCGN1298 cDNA Clones

DNA Sequencing

The polyA⁺ sequence was missing from pCGN1299 cDNA. A longer cDNA clone, pCGN1298, therefore was identified by its hybridization with the pCGN488 probe. The complete DNA sequence of the two cDNA inserts was determined using both Maxam-Gilbert and the Sanger di-deoxy techniques and is as follows. The sequence of pCGN1298 contains additional sequences at both the 5' and 3' end compared to pCGN1299. As shown in Figure 1, the sequences are identical over the region that the two clones have in common.

Amino Acid Sequence

The pCGN1299 cDNA sequence was translated in three frames. The longest open reading frame (which starts from the first ATG) is indicated. Both pCGN1299 and pCGN1298 have an open reading frame which encodes a

96 amino acid polypeptide (see Figure 1). The protein has a hydrophobic N-terminus which may indicate a leader peptide for protein targeting. A hydrophobicity profile was calculated using the Hopp and Woods, (Proc. Natl. Acad. Sci. USA (1981) 78:3824-3828) algorithm. Residues 10-23 have an extremely hydrophobic region. A comparison of 2A11 to pea storage proteins and other abundant storage proteins is shown in Figure 2. The sulfur-rich composite of the fruit-specific protein is similar to a pea storage protein which has recently been described (see Higgins et al., J. Biol. Chem. (1986) 261:11124-11130, for references to the individual peptides). This may indicate a storage role for this fruit-specific protein abundant species.

Example 4

Screening Genomic Library for Genomic Clones

Southern Hybridization

Southern analysis was performed as described by Maniatis et al., 1982. Total tomato DNA from cultivar UC82B was digested with EcoRI or HindIII, separated by agarose gel electrophoresis and transferred to nitrocellulose. Southern hybridization was performed using a ^{32}P -labeled probe produced by nick translation of pCGN488 (Maniatis et al., 1982). The simple hybridization pattern indicated that the gene encoding pCGN1299 cDNA was present in a few or perhaps even one copy in a tomato genome.

Isolation of a Genomic Clone

A genomic library established in Charon35/Sau3A constructed from DNA of the tomato cultivar VFNT-Cherry was screened using the [^{32}P]-RNA from cDNA clone pCGN488 as a probe. A genomic clone containing approximately 12.5 kb of sequence from the tomato genome was isolated. The region which hybridizes to a pCGN488

probe spans an XbaI restriction site which was found in the cDNA sequence and includes the transcriptional initiation region designated 2A11.

5 Sequence of Genomic Clone

The DNA sequence of the genomic clone was determined by Sanger dideoxy techniques and is as shown in Figure 4. The sequence of the genomic clone is identical to the pCGN1299 cDNA clone over the region
10 they have in common.

Subcloning

The region surrounding the XbaI restriction site, approximately 2.4 kb in the 5' direction and approximately 2.1 kb in the 3' direction was subcloned to
15 provide an expression cassette. The 5' XhoI to XbaI fragment and the 3' XbaI to EcoRI fragment from the 2A11 genomic clone were inserted into a pUC-derived chloromphenicol plasmid containing a unique XhoI site
20 and no XbaI site. This promoter cassette plasmid is called pCGN1273.

Example 5

Construction of Fruit-

25 Specific Antisense Cassette

Insertion of Antisense Fragment

The 2A11 genomic fragment was tagged with PG antisense sequences by insertion of PG into the unique XbaI site of the pCGN1273 promoter cassette in the anti-
30 sense orientation. The inserted sequences increased the size of the mRNA over the endogenous transcript, and thus the expression pattern of the construct could be compared to the endogenous gene by a single Northern hybridization in a manner analogous to the detection of
35 a tuber-specific potato gene described by Eckes et al., Mol. Gen. Genet. 1986 205:14-22.

Example 6Insertion of Tagged Genomic Construction
Into Agrobacterium Binary Vectors

The tagged genomic construction is excised
5 using the flanking XhoI restriction enzyme sites and is
cloned into the unique SalI site of the binary plasmid
pCGN783 containing a plant kanamycin resistance marker
between the left and right borders to provide plasmid
pCGN1269.

10 This plasmid binary vector in E. coli C2110 is
conjugated into A. tumefaciens containing a disarmed
Ti-plasmid capable of transferring the polygalacturo-
nase antisense cassette and the kanamycin resistance
cassette into the plant host genome.

15 The Agrobacterium system which is employed is
A. tumefaciens PC2760 (G. Ooms et al., Plasmid (1982)
7:15-29; Hoekema et al., Nature (1983) 303:179-181;
European Patent Application 84-200239.6, 2424183).

20 1. Construction of pCGN783

pCGN783 is a binary plasmid containing the
left and right T-DNA borders of A. tumefaciens octopine
Ti-plasmid pTiA6 (Currier and Nester, J. Bacteriol.
(1976) 126:157-165) the gentamicin resistance gene of
25 pPH1J1 (Hirsch et al., Plasmid (1984) 12:139-141), the
35S promoter of cauliflower mosaic virus (CaMV)
(Gardner et al., Nucleic Acid Res. (1981) 9:1871-1880);
the kanamycin resistance gene of Tn5 (Jorgensen, Mol.
Gen. (1979) 177:65); and the 3' region from transcript
30 7 of pTiA6 (Currier and Nester, supra (1976)). A sche-
matic diagram of the construction of pCGN783 is shown
in Figure 3. (a) through (f) refer to the plasmid
constructions detailed below.

(a) Construction of pCGN587

The HindIII-SmaI fragment of Tn5 containing the entire structural gene for APH3'II (Jorgensen et al., Mol. Gen. (1979) 177:65), was cloned into pUC8 (Vieira and Messing, Gene (1982) 19:259), converting the fragment into a HindIII-EcoRI fragment, since there is an EcoRI site immediately adjacent to the SmaI site. The PstI-EcoRI fragment containing the 3' portion of the APH3'II gene was then combined with an EcoRI-BamHI-SalI-PstI linker into the EcoRI site of pUC7 (pCGN546W). Since this construct does not confer kanamycin resistance, kanamycin resistance was obtained by inserting the BglI-PstI fragment of the APH3'II gene into the BamHI-PstI site (pCGN546X). This procedure reassembles the APH3'II gene, so that EcoRI sites flank the gene. An ATG codon was upstream from and out of reading frame with the ATG initiation codon of APH3'II. The undesired ATG was avoided by inserting a Sau3A-PstI fragment from the 5' end of APH3'II, which fragment lacks the superfluous ATG, into the BamHI-PstI site of pCGN546W to provide plasmid pCGN550. The EcoRI fragment of pCGN550 containing the APH3'II gene was then cloned into the EcoRI site of pUC8-pUC13 (K. Buckley supra (1985)) to give pCGN551.

Each of the EcoRI fragments containing the APH3'II gene was then cloned into the unique EcoRI site of pCGN451, which contains an octopine synthase cassette for expression to provide pCGN548 (2ATG) and pCGN552 (1ATG). The plasmid pCGN451 having the ocs 5' and the ocs 3' in the proper orientation was digested with EcoRI and the EcoRI fragment from pCGN551 containing the intact kanamycin resistance gene inserted with EcoRI site to provide pCGN552 having the kanamycin resistance gene in the proper orientation. This ocs/KAN gene was used to provide a selectable marker for the trans type binary vector pCGN587.

The 5' portion of the engineered octopine synthase promoter cassette consists of pTiA6 DNA from the XhoI at bp 15208-13644 (Barker et al., supra (1983)), which also contains the T-DNA boundary sequence (border) implicated in T-DNA transfer. In the plasmid pCGN587, the osc/KAN gene from pCGN552 provides a selectable marker as well as the right border. The left boundary region was first cloned in M13mp9 as a HindIII-SmaI piece (pCGN502) (base pairs 602-2212) and recloned as a KpnI-EcoRI fragment in pCGN565 to provide pCGN580. pCGN565 is a cloning vector based on pUC8-Cm, but containing pUC18 linkers. pCGN580 was linearized with BamHI and used to replace the smaller BglI fragment of pVCK102 (Knauf and Nester, Plasmid (1982) 8:45), creating pCGN585. By replacing the smaller SalI fragment of pCGN585 with the XhoI fragment from pCGN552 containing the ocs/KAN gene, pCGN587 was obtained.

(b) Construction of pCGN739 (Binary Vector)

To obtain the gentamicin resistance marker, the resistance gene was isolated from a 3.1 kb EcoRI-PstI fragment of pPHIJI (Hirsch et al., Plasmid (1984) 12:139-141) and cloned into pUC9 (Vieira et al., Gene (1982) 19:259-268) yielding pCGN549.

The pCGN549 HindIII-BamHI fragment containing the gentamicin resistance gene replaced the HindIII-BglII fragment of pCGN587 (for construction, see 6.1(a), supra) creating pCGN594.

The pCGN594 HindIII-BamHI region which contains an ocs-kanamycin-ocs fragment was replaced with the HindIII-BamHI polylinker region from pUC18 (Yanisch-Perron, Gene (1985) 33:103-119) to make pCGN739.

(c) Construction of 726c (1 ATG-Kanamycin-3' region)

pCGN566 contains the EcoRI-HindIII linker of pUC18 (Yanisch-Perron, ibid) inserted into the EcoRI-HindIII sites of pUC13-Cm (K. Buckley, Ph.D. Thesis,

University of California, San Diego, 1985). The HindIII-BglII fragment of pNW31c-8, 29-1 (Thomashow et al., Cell (1980) 19:729) containing ORF1 and 2 (Barker et al., Plant Mol. Biol. (1984) 2:335-350) was subcloned into
 5 the HindIII-BamHI sites of pCGN566 producing pCGN703.

The Sau3A fragment of pCGN703 containing the 3' region of transcript 7 from pTiA6 (corresponding to bases 2396-2920 of pTi15955 (Barker et al., supra (1984))) was subcloned into the BamHI site of pUC18 (Yanisch-Perron et al., supra (1985)) producing pCGN709.
 10

The EcoRI-SmaI polylinker region of pCGN709 was replaced with the EcoRI-SmaI fragment from pCGN587 (see 6.1(a), supra) which contains the kanamycin resistance gene (APH3'II) producing pCGN726.

The EcoRI-SalI fragment of pCGN726 plus the BglII-SalI sites of pUC8-pUC13-cm (chloramphenicol resistant, K. Buckley, Ph.D. Thesis, University of California, San Diego, 1985) producing pCGN738. To construct pCGN734, the HindIII-SphI site of M13mp19 (Norrandar et al., Gene (1983) 26:101-106). Using an oligonucleotide corresponding to bases 3287 to 3300, DNA synthesis was primed from this template. Following S1 nuclease treatment and HindIII digestion, the resulting fragment was cloned into the HindIII-SmaI site of pUC19 (Yanisch-Perron et al., supra (1985)). The resulting EcoRI to HindIII fragment of pTiA6 (corresponding to bases 3390-4494) into the EcoRI site of pUC8 (Vieira and Messing, supra (1982)) resulting in pCGN734. pCGN726c is derived from pCGN738 by deleting the 900 bp
 20
 25
 30 EcoRI-EcoRI fragment.

(d) Construction of pCGN167

pCGN167 is a construct containing a full length CamV promoter, 1 ATG-kanamycin gene, 3' end and the
 35 bacterial Tn903-type kanamycin gene. MI is an EcoRI fragment from pCGN550 (see construction of pCGN587) and was cloned into the EcoRI cloning site in the 1 ATG-

kanamycin gene proximal to the polylinker region of M13mp9. See copending Application Serial No. 920,579, filed October 17, 1986, which disclosure is incorporated herein by reference.

5 To construct pCGN167, the AluI fragment of CamV (bp 7144-7735) (Gardner et al., Nucl. Acids Res. (1981) 9:2871-2888) was obtained by digestion with AluI and cloned into the HincII site of M13mp7 (Vieira, Gene (1982) 19:259) to create C614. An EcoRI digest of C614
10 produced the EcoRI fragment from C614 containing the 35S promoter which was cloned into the EcoRI site of pUC8 (Vieira et al., Gene (1982) 19:259) to produce pCGN146. To trim the promoter region, the BglII site (bp 7670) was treated with BglII and Bal31 and subse-
15 quently a BglII linker was attached to the Bal31 treated DNA to produce pCGN147.

pCGN148a containing the promoter region, selectable marker (KAN with 2 ATGs) and 3' region was prepared by digesting pCGN528 (see below) with BglII
20 and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment was cloned into the BglII site of pCGN528 so that the BglII site was proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, was made as follows. pCGN525 was made by
25 digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et al., Mol. Gen. (1979) 177:65) with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the kanamycin gene into the
30 HindIII-BamHI sites in the tetracycline gene of PACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156). pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et al., Cell (1980) 19:729-739) into the BamHI site of pCGN525. pCGN528
35 was obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

pCGN149a was made by cloning the BamHI kana-
mycin gene fragment from pMB9KanXXI into the BamHI site
of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and
Messing, Gene (1982) 19:259-268) which has the XhoI site
5 missing but contains a functional kanamycin gene from
Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a was digested with BglII and SphI.
This small BglII-SphI fragment of pCGN149a was replaced
with the BamHI-SphI fragment from MI (see below) iso-
10 lated by digestion with BamHI and SphI. This produces
pCGN167.

(e) Construction of pCGN766c (35S promoter-3' region)

The HindIII-BamHI fragment of pCGN167 contain-
15 ing the CaMV-35S promoter, 1 ATG-kanamycin gene and the
BamHI fragment 19 of pTiA6 was cloned into the BamHI-
HindIII sites of pUC19 (Norranders et al., supra (1985);
Yanisch-Perron et al., supra (1985)) creating pCGN976.

The 35S promoter and 3' region from transcript
20 7 was developed by inserting a 0.7 kb HindIII-EcoRI
fragment of pCGN976 (35S promoter) and the 0.5 kb
EcoRI-SalI fragment of pCGN709 (transcript 7:3' for
construction see supra) into the HindIII-SalI sites of
pCGN566 creating pCGN766c.

25 (f) Final Construction of pCGN783

The 0.7 kb HindIII-EcoRI fragment of pCGN766c
(CaMV-35S promoter) was ligated to the 1.5 kb EcoRI-
SalI fragment of pCGN726c (1-ATG-KAN-3' region) into
30 the HindIII-SalI sites of pUC119 (J. Vieira, Rutgers
University, New Jersey) to produce pCGN778. The 2.2 kb
region of pCGN778, HindIII-SalI fragment containing the
CaMV 35S promoter (1-ATG-KAN-3' region) replaced the
HindIII-SalI polylinker region of pCGN739 to produce
35 pCGN783.

Example 7
Transfer of Genomic Construction
to Tomato via Cocultivation

Substantially sterile tomato cotyledon tissue is obtained from seedlings which have been grown at 24°C, with a 16hr/8hr day/night cycle in 100x25 mm petri dishes containing Murashige-Skoog salt medium and 0.8% agar (pH 6.0). Any tomato species may be used, however, here the inbred breeding line was UC82B, available from the Department of Vegetable Crops, University of California, Davis, CA 95616. The cotyledons are cut into three sections and the middle placed onto feeder plates for a 24-hour preincubation. The feeder plates are prepared by pipetting 0.5 ml of a tobacco suspension culture (10^6 cells/ml) onto 0.8% agar medium, containing Murashige minimal organic medium (K.C. Biologicals), 2,4-D (0.1 mg/l), kinetin (1 mg/l), thiamine (0.9 mg/l) and potassium acid phosphate (200 mg/l, pH 5.5). The feeder plates are prepared two days prior to use. A sterile 3 mm filter paper disk containing feeder medium is placed on top of the tobacco cells after the suspension cells are grown for two days.

Following the preincubation period, the middle one third of the cotyledon sections are placed into a liquid MG/L broth culture (1-5 ml) of the A. tumefaciens strain. The binary plasmid pCGN1269 is transferred to A. tumefaciens strain 2760 by conjugation or by transformation selecting for Gentamicin resistance encoded by the plasmid pCGN1269. The cotyledon sections are cocultivated with the bacteria for 48 hrs on the feeder plates and then transferred to regeneration medium containing 500 mg/l carbenicillin and 100 mg/l kanamycin. The regeneration medium is a K.C. Biologicals Murashige-Skoog salts medium with zeatin (2 mg/l) myo-inositol (100 mg/l), sucrose (20 g/l), Nitsch vitamins and containing 0.8% agar (pH 6.0). In 2-3 weeks, shoots are observed to develop. When the shoots are

approximately 1.25 cm, they are excised and transferred to a Murashige and Skoog medium containing carbenicillin (500 mg/l) and kanamycin (50 mg/l) for rooting. Roots develop within 10-12 days.

Shoots which develop and subsequently root on media containing the kanamycin are tested for APH3'II enzyme.

An aminoglycoside phosphotransferase enzyme (APH3'II) assay is conducted on putative transformed tomato plants and shoots. APH3'II confers resistance to kanamycin and neomycin. APH3'II activity is assayed (Reiss *et al.*, *Gene* (1984) 30:211-218) employing electrophoretic separation of the enzyme from other interfering proteins and detection of its enzymatic activity by *in situ* phosphorylation of kanamycin. Both kanamycin and [γ - 32 P] ATP act as substrates and are embedded in an agarose gel which is placed on top of the polyacrylamide gel containing the proteins. After the enzymatic reaction, the phosphorylated kanamycin is transferred to P-81 phosphocellulose ion exchange paper and the radiolabeled kanamycin is finally visualized by autoradiography. The Reiss *et al.* method is modified in the final washing of the P-81 ion exchange paper by rinsing in 0.1 mg/ml of proteinase K.

Example 8

Construction of Tagged 2A11 Plasmids

In Binary Vectors

The complete sequence of the 2A11 genomic DNA cloned into pCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654) is shown in Figure 4.

pCGN1267 was constructed by deleting from pCGN1273 a portion of the plasmid polylinker from the EcoRV site to the BamHI site. Two DNA sequences were inserted into pCGN1273 at the unique XbaI site (position 2494). This site is in the 3' non-coding region of the 2A11 genomic clone before the poly A site.

pCGN1273 was tagged with 360 bp (from base number 1 to 360) from the 5' region of the tomato polygalacturonase (PG) cDNA clone, F1 (Sheehy *et al.*, *Mol. Gen. Genet.* (1987) 208:30-36) at the unique XbaI restriction enzyme site. The tag was inserted in the antisense orientation resulting in plasmid pCGN1271 and in the sense orientation yielding plasmid pCGN1270. Each plasmid was linearized at the unique BglII restriction enzyme site and cloned into the binary vector pCGN783 at the unique BamHI restriction enzyme site.

pCGN1273 was also tagged with a 0.5 kb fragment of DNA (base number 1626 to 2115) from a PG genomic clone (see Figure 5) which spans the 5' end of the intron/exon junction. This fragment was cloned into the XbaI site resulting in plasmid pCGN1215. pCGN1215 was linearized at the unique BglII site and cloned into pCGN783 at the BamHI site resulting in two plasmids, pCGN1219 and pCGN1220, which differ only in the orientation of pCGN1215 within pCGN783.

Three DNA sequences were inserted into pCGN1267 at the unique ClaI sites (position 2402, 2406). These sites are in the 3' non-coding region of the 2A11 genomic clone, 21 bp from the stop codon. The 383 bp XbaI fragment from the PG cDNA clone was cloned into the ClaI site of pCGN1267 after filling in the XbaI and ClaI ends with Klenow and blunt ligation. The fragment in a sense orientation resulted in plasmid pCGN1263 and in the antisense orientation gave pCGN1262. pCGN1263 was linearized at the unique BglII site and cloned into pCGN783 at the BamHI site yielding pCGN1260. pCGN1262 was also linearized at the BglII site and cloned into pCGN783 at the BamHI site resulting in two plasmids, pCGN1255 and pCGN1258, which differ only in the orientation of pCGN1262 in the binary vector pCGN783.

The 0.5 kb fragment of the PG genomic clone spanning the intron/exon junction (*supra*) was cloned into pCGN1267 at the ClaI site in an antisense direc-

tion yielding plasmid pCGN1225. This plasmid was linearized at the BglII restriction enzyme site and cloned into pCGN783 at the BamHI site producing two plasmids, pCGN1227 and pCGN1228, which differ only in the orientation of pCGN1225 in the binary vector.

The Eco7 fragment (base numbers 5545 to 12,823) (Barker et al., Plant Mol. Biol. (1983) 2:335-350) from the octopine plasmid pTiA6 of A. tumefaciens (Knauf and Nester, Plasmid (1982) 8:45-54) was subcloned into pUC19 at the EcoRI site resulting in plasmid pCGN71. A RsaI digest allowed a fragment of DNA from bases 8487 to 9036 of the Eco7 fragment to be subcloned into the vector ml3 BlueScript Minus (Stratagene, Inc.) at the SmaI site resulting in plasmid pCGN1278. This fragment contains the coding region of the genetic locus designated tmr which encodes a dimethylallyl transferase (isopentenyl transferase) (Akiyoshi et al., Proc. Natl. Acad. Sci. USA (1984) 81:5994-5998; Barry et al., ibid (1984) 81:4776-4780). An exonuclease/mung bean treatment (Promega Biotech) produced a deletion on the 5' end of the tmr gene to a point 39 base pairs 5' of the start codon. The tmr gene from pCGN1272 was subcloned into the ClaI site of pCGN1267. The tmr gene in the sense orientation yielded pCGN1261 and in the antisense orientation gave plasmid pCGN1266. pCGN1261 was linearized at the BglII site and cloned into pCGN783 at the BamHI site resulting in plasmid pCGN1254. pCGN1266 was also linearized at the BglII site and subcloned into pCGN783 at the BamHI site yielding two plasmids, pCGN1264 and pCGN1265, which differ only in the orientation of pCGN1266 in pCGN783.

Analysis of Expression in Transgenic Plants

Immature green fruit (approximately 3.2 cm in length) was harvested from two tomato plants cv. UC82B that had been transformed with a disarmed Agrobacterium

strain containing pCGN1264. Transgenic plants are designated 1264-1 and 1264-11. The pericarp from two fruits of each plant was ground to a powder under liquid N₂, total RNA extracted and polyA⁺ mRNA isolated (as described in Mansson *et al.*, *Mol. Gen. Genet.* (1985) 200:356-361). Young green leaves were also harvested from each plant and polyA⁺ mRNA isolated.

Approximately 19 µg of total RNA from fruit, 70 ng of polyA⁺ mRNA from fruit and 70 ng of polyA⁺ mRNA from leaves from transformed plants 1264-1 and 1264-11 was run on a 0.7% agarose formaldehyde Northern gel and blotted onto nitrocellulose (Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982) Cold Spring Harbor, New York). Also included on the gel as a negative control was approximately 50 ng of polyA⁺ mRNA from leaf and immature green fruit of a nontransformed UC82B plant.

As a positive control and to help in quantitating mRNA levels, *in vitro* transcribed RNA from pCGN1272 was synthesized using T3 polymerase (Stratagene, Inc.). Nineteen pg and 1.9 pg of this *in vitro* synthesized RNA were loaded on the Northern gel.

The probe for the Northern filter was the 1.0 kb *tmr* insert DNA (a *Kpn*I to *Sac*I fragment) from pCGN1272 isolated by electroelution from an agarose gel (Maniatis, *supra* (1982)) and labeled by nick translation (Bethesda Research Laboratory kit) using α³²P dCTP (Amersham).

The Northern filter was prehybridized at 42°C for 5 hrs in the following solution: 25 ml formamide, 12.5 ml 20X SSC, 2.5 ml 1 M NaP, 5 ml 50X Denhardt's, 0.5 ml 10% SDS, 1 ml 250 mM EDTA, 1 ml 10 mg/ml ssDNA and 2 ml H₂O. Then one-fifth volume of 50% dextran sulfate and approximately 2.2X 10⁷ cpm of the probe was added and hybridization was for 15 hrs at 42°C.

The Northern filter was washed one time in 2X SSC and 0.1% SDS at 55°C for 20 minutes each wash. The filter was allowed to air dry before being placed with Kodak XAR film and an intensifying screen at -70° for two days.

Northern Results on Transgenic Plants

The nicked tmr probe hybridized with a mRNA species approximately 1.7 kb in length was observed in the total RNA and polyA⁺ mRNA fruit lanes of the Northern blot. This is the expected length of the reintroduced 2All gene (0.7 kb) tagged with the tmr gene (1.0 kb) in the antisense orientation. The level of expression from the reintroduced tagged gene is somewhat lower than the level of expression of the endogenous 2All gene. The level of expression of the reintroduced gene in immature green fruit is higher than the expression level in leaf tissue with a small amount of hybridizing mRNA in leaf tissue in these transformants.

Example 9

Screening Genomic Library for Polygalacturonase Genomic Clones

Isolation of a Genomic Clone

An EcoRI partial genomic library established in Charon 4 constructed from DNA of a Lycopersicon esculentum cultivar was screened using a probe from the polygalacturonase cDNA (Sheehy et al., Mol. Gen. Genet. (1987) 208:30-36). A lambda clone containing an approximately 16 kb insert was isolated from the library, of which an internal 2207 bp HindIII to EcoRI was sequenced. The HindIII-EcoRI fragment includes the polygalacturonase promoter region.

Sequence of Genomic Clone

The DNA sequence of the genomic clone was determined by Sanger dideoxy techniques and is as shown in Figure 5. The sequence of the genomic clone bases 1427 to 1748 are homologous to the polygalacturonase cDNA sequence.

The above results demonstrate the ability to identify inducible regulatory sequences in a plant genome, isolate the sequences and manipulate them. In this way, the production of transcription cassettes and expression cassettes can be produced which allow for differentiated cell production of the desired product. Thus, the phenotype of a particular plant part may be modified, without requiring that the regulated product be produced in all tissues, which may result in various adverse effects on the growth, health, and production capabilities of the plant. Particularly, fruit-specific transcription initiation capability is provided for modifying the phenotypic properties of a variety of fruits to enhance properties of interest such as processing, organoleptic properties, storage, yield, or the like.

E. coli strain pCGN1299x7118 was deposited with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 21, 1987 and given Accession No. 67408.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may
5 be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A DNA construct comprising in the direction of transcription, a fruit-specific transcriptional initiation region from a gene expressed at or immediately after anthesis or at the breaker stage, said gene remaining expressed at least until the ripe period, joined to a DNA sequence of interest other than the wild-type sequence associated with said initiation region, wherein said DNA sequence of interest is under the transcriptional regulation of said initiation region, and a transcriptional termination region.

2. A DNA construct according to Claim 1, wherein said transcriptional initiation region is from a gene expressed immediately upon anthesis.

3. A DNA construct according to Claim 1, wherein said transcriptional initiation region regulates transcription of a gene encoding a plant storage protein.

4. A DNA construct according to Claim 3, wherein said transcriptional initiation region is the 2A11 region.

5. A DNA construct according to Claim 1, wherein said DNA sequence of interest is a sequence complementary to a native plant transcript.

6. A DNA construct according to Claim 1, wherein said DNA sequence of interest is an open reading frame encoding an amino acid sequence of interest.

7. A DNA construct according to Claim 1, wherein said DNA sequence of interest is a polygalacturonase gene or fragment thereof of at least 12nt in the anti-sense direction.

8. A DNA construct for integration into a plant genome comprising at least the right T-DNA border joined to a DNA construct according to Claim 1.

5

9. A DNA construct comprising in the direction of transcription, the fruit-specific transcriptional initiation region of a plant storage protein being active at or immediately after anthesis and remaining active until at least until the ripe period, joined to a DNA sequence other than the wild-type sequence, wherein said sequence comprises a unique restriction site for insertion of a sequence of interest to be under the transcriptional regulation of said initiation region, and a transcriptional termination region.

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10. A DNA construct according to Claim 9, wherein said transcriptional initiation region is the 2A11 region.

20

11. A DNA construct for integration into a plant genome comprising at least the right T-DNA border joined to a DNA construct according to Claim 10.

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12. A DNA vector comprising a broad spectrum prokaryotic replication system and a DNA construct according to Claim 1.

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13. A DNA vector comprising a broad spectrum prokaryotic replication system and a DNA construct according to Claim 9.

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14. A method for specifically modifying the phenotype of fruit substantially distinct from other plant tissue, said method comprising:

transforming a tomato plant cell with a DNA construct under genomic integration conditions, wherein said DNA construct comprises in the direction of transcription, a 2A11 fruit-specific transcriptional initiation region, joined to a DNA polygalacturonase gene sequence, wherein said sequence is oriented in the antisense direction and under the transcriptional regulation of said initiation region, and a transcriptional termination region, whereby said DNA construct becomes integrated into the genome of said plant cell, whereby said antisense sequence is transcribed and inhibits expression of polygalacturonase in fruit;
regenerating a plant from said transformed plant cell; and
growing said plant to produce fruit of the modified phenotype.

15. A method according to Claim 14, wherein said transcription initiation region is the 2A11 region.

16. A plant cell comprising a DNA construct according to Claim 1:

17. A plant cell comprising a DNA construct according to Claim 9:

18. A method for specifically modifying the phenotype of tomato fruit substantially distinct from other plant tissue, said method comprising:
transforming a plant cell with a DNA construct under genomic integration conditions, wherein said DNA construct comprises in the direction of transcription, a fruit-specific transcriptional initiation region being active at or immediately after anthesis, said gene remaining active at least until the ripe period, joined to a DNA sequence other than the wild-type sequence and capable of modifying the phenotype of fruit cells upon

transcription, wherein said sequence is under the transcriptional regulation of said initiation region, and a transcriptional termination region, whereby said DNA construct becomes integrated into the genome of said
5 plant cell;

regenerating a plant from said transformed plant cell; and

growing said plant to produce fruit of the modified phenotype.

10

19. A plant comprising a DNA construct according to Claim 1.

15 20. A plant comprising a DNA construct according to Claim 9.

21. Fruit comprising a construct according to Claim 1.

20 22. Fruit according to Claim 21, wherein said fruit is tomato.

25 23. Fruit according to Claim 22, wherein said DNA sequence of interest is a polygalacturonase gene or fragment of at least 12nt thereof oriented in the anti-sense direction and said transcsription initiation region is 2A11.

30 24. Fruit according to Claim 21, wherein said transcription initiation region is 2A11.

3H11 TTTTTTTGAGCAAAGGGCAACTCAGATATCCAAAGATGAATCCAACATATA 51

3H11 GCTTACAGCTGGGAGAACATTGTCTAACTCTTCTGAAATTTAAATGTTATC 102

3H11 CAGAATCCTTCATCATAAAATAATATCAAAATGCAAATCTATTTTTTCTAC 153

3H11 TCTTGTCTAGCTTCAACTTTCTTCTTCTGCTCATCAATTAGCAATTAATCC 204
 TGCTCATCAATTAGCAATTAATCC

3H11 AAAACCATTATGGCTGCCAAAAATTCAGAGATGAAGTTTGCTATCTTCTTC 255
 2A11 AAAACCATTATGGCTGCCAAAAATTCAGAGATGAAGTTTGCTATCTTCTTC
 METAlaAlaLysAsnSerGluMETLysPheAlaIlePhePhe

3H11 GTTGTTCCTTTTGACGACCACTTTAGTTGATATGTCTGGAATTCGAAAATG 306
 2A11 GTTGTTCCTTTTGACGACCACTTTAGTTGATATGTCTGGAATTCGAAAATG
 ValValLeuLeuThrThrThrLeuValAspMETSerGlyIleSerLysMET

3H11 CAAGTGATGGCTCTTCGAGACATACCCCCACAAGAAACATTGCTGAAAATG 357
 2A11 CAAGTGATGGCTCTTCGAGACATACCCCCACAAGAAACATTGCTGAAAATG
 GlnValMETAlaLeuArgAspIleProProGlnGluThrLeuLeuLysMET

3H11 AAGCTACTTCCCACAAATATTTTGGGACTTTGTAACGAACCTTGCAGCTCA 408
 2A11 AAGCTACTTCCCACAAATATTTTGGGACTTTGTAACGAACCTTGCAGCTCA
 LysLeuLeuProThrAsnIleLeuGlyLeuCysAsnGluProCysSerSer

3H11 AACTCTGATTGCATCGGAATTACCCTTTGCCAATTTTGTAAAGGAGAAGACG 459
 2A11 AACTCTGATTGCATCGGAATTACCCTTTGCCAATTTTGTAAAGGAGAAGACG
 AsnSerAspCysIleGlyIleThrLeuCysGlnPheCysLysGluLysThr

3H11 GACCAGTATGGTTTAAACATACCGTACATGCAACCTGTTGCCTTGAACAATA 510
 2A11 GACCAGTATGGTTTAAACATACCGTACATGCAACCTGTTGCCTTGAACAATA
 AspGlnTyrGlyLeuThrTyrArgThrCysAsnLeuLeuPro .

FIG. 1-1

3H11 TCAATGATCTATCGATCGATCTATCTATCTATTTATCTGTCTCTGCGCGTA 561
2A11 TCAATGATCTATCGATCGATCTATCTATCTATTTATCTGTCTCTGCGCGTA

3H11 TAGTGTTGTCTGTACCTTTGGTGTGAAGAATATGAATAAAGGGATACATAT 612
2A11 TAGTGTTGTCTGTACCTTTGGTGTGAAGAATATGAATAAAGGGATACATAT

3H11 ATCTAGATATATTCTAGGTAATGTCCTATTGTATTTAAAATTTGTAGCAAT 663
2A11 ATCTAGATATATTCTAGGTAATGTCCTATTGTATTTAAAATTTGTAGCAAT

3H11 GATTGTTTGAATAAAAACATACCATGAGTGAAATAATTATTCCACATTAAT 714
2A11 GATTGTTTGAATAAAAACATACCATGAGTGAAATAATTATTCC

3H11 TCACGTATTTATTTCACTTATGATACGTATTTTTGTTCCTTTGCGGTAAAA 765

3H11 AAAAAAAAAA 774

FIG. 1-2

FIG. 2-1

2A11	⑤MALRDIPPQETLL
PA1b	⑤CSPFDIPPCGSPLCRCI
Chick pea inhibitor	⑤CT-KSIPP-----QCRCN
Lima bean inhibitor	LCT-KSIPP-----QCRCT
α_1 -antitrypsin	LGAIPMSIPPEV

FIG. 2-2

2A11	TNHLGLCNEPCSSNSDCT
PA1b	GSPLCRICIPAGLVIGNCR
Barley chloroform/ methanol-soluble protein d	TNLLGNCR-FYLVQQTCA
Wheat α -amylase inhibitor 0.28	VSALTGCR-AMVKLQ-CV
Wheat albumin	VPALPACRPL-LRLQ-CN
Millet bi-functional inhibitor	NNPLDSCRWYVS _A T _K R _T ACG
Castor bean 2S small subunit	QQNLRQCQEYIKQQVSGQ
Napin small subunit	AQNLRACQQWLNKQAMQS



Fig. 3

2A11 GENOMIC

10	20	30	40	50
CTCGAGCCCT	TTAAAAAGTA	TAGTCAATAT	TTACGGTGAC	CGTGAATTTT
60	70	80	90	100
TTAATTATGA	TATATAATTT	AAAAGAAATC	ATGATCACAT	TCTACTGATG
110	120	130	140	150
AGAACATGTG	CTAATCAAGG	GAAAACATGG	ATGTGAAAAA	TACTTTTTGT
160	170	180	190	200
TAAAAGTAAA	AAAAAATGTG	AAATTTTGTT	AGTTATTTAC	TACCTATACA
210	220	230	240	250
TTATTTGAGC	ATGTGCAAAC	TTTACAAATA	CCTAATAGAA	GATTTTCACC
260	270	280	290	300
TGCCTGTATA	TATGTAAATT	AATTATAATG	AACACTCTCA	CATAAAATAA
310	320	330	340	350
TTATCAGTAT	ATACATTAAT	ACTTGCCCTC	CACAATGAAT	TAAATAAAAT
360	370	380	390	400
GTAGAACATG	ATCTACACTT	CAATAAAACT	AAGACCATAA	AGAATAATTT
410	420	430	440	450
CAAAATATAC	ACATGTCAAC	AATAAATTAT	TTGCATATTA	TATTAACCTA
460	470	480	490	500
CTAAACAATC	TTTACTTTTG	AAATATAAAA	ATAATCAAGT	TATAAGTCTG
510	520	530	540	550
CTCAAAGTAA	AGCACTTGTT	AGACTCATCT	GATTTTGAGA	AGGTAAGCAA
560	570	580	590	600
ATTGATGGTG	CATAATAGTC	ACAAGTAAAA	TATAAAATAG	ATTTCATTAG
610	620	630	640	650
TAAAATTGTT	TTTTACTTTC	TTTATATATA	ATTATCAATA	TCCTTCAATG
660	670	680	690	700
GTAGGTTAAT	TATATTGTTA	ACTTCTTGTT	GAATTAAAGC	AATAAGACAA
710	720	730	740	750
GAATATTAAA	GATAAAAGAA	CAATAAAAAT	AGAAAGACTA	AGAGATAAGA
760	770	780	790	800
GTTTTCTTAT	TCTTCTTTCA	ATAAGTATCA	TCAAGTGTAT	ACAATATAAA
810	820	830	840	850
TTTTTGTATT	TTTGATCTAT	CTATTTATAA	TGTTATATAT	AAGCATACAA
860	870	880	890	900
AAGATCAGTC	ATAAATATGA	CTTTAATCAT	GAAAATAATG	AAAGAGATTA
910	920	930	940	950
TGAAGGCGTA	AGGTACTAG	AATAATAGTC	ATTAAAAAAA	GGGGTTATCT
960	970	980	990	1000
TTATAATTGA	ATAATTGATG	AAGTAATGGA	GATAATTAGT	GAGCATAAAT
1010	1020	1030	1040	1050
TTTTTTAAAA	AAATGGACAT	TTACACTATA	ATATTTTATA	ACACTTCC
1060	1070	1080	1090	1100
TTAAACATCT	AGGTATAAAT	AATGAGTCTT	GTCAAAATCT	TAGTAGGAAA

FIG. 4-1

1110	1120	1130	1140	1150
AATTCTGTGA	AATTTTTTTTA	GTGAAAACAA	ATGATATAAA	TATCTTGAAT
1160	1170	1180	1190	1200
ACTCATTATT	TGTTGTCTCA	TTAAAAATCT	TATCTGACCT	ATAAAATAAA
1210	1220	1230	1240	1250
TTATTTGCTC	AACTCAAAAT	AGTTTTTTCAT	TCTAAAATTA	GTATAATTAT
1260	1270	1280	1290	1300
TAGTGAATAT	TTAATTAACA	TAATTGTATA	CTAAGGGGCC	TATAAATTGG
1310	1320	1330	1340	1350
ATTCTTCTCA	AAGAAAAATA	AAATCACCAC	ACAACTTTCT	TCTTCTGCTC
1360	1370	1381	1390	
ATCAATTAGC	AATTAATCCA	AAACCATT	ATG GCT GCC AAA AAT	
			MET Ala Ala Lys Asn	
1399	1408	1417	1426	
TCA GAG ATG	AAG TTT GCT	ATC TTC TTC	GTT GTT CTT	TTG
Ser Glu MET	Lys Phe Ala	Ile Phe Phe	Val Val Leu	Leu
1435	1444	1454	1464	1474
ACG ACC ACT	TTA GGTTCACAAC	ACTTCTCCCT	TATTTTGT	
Thr Thr Thr	Leu			
1484	1494	1504	1514	1524
TCTTAATTTT	TTGGAAGTCA	TATGCATGTG	TTTGGTATCA	TGGTATATAT
1534	1544	1554	1564	1574
ATAAAGGAAA	ATATTTTTCT	TAATTACTGG	TTTTCTAATG	TTTGGTAGGT
1584	1594	1604	1614	1624
AATCGGAAAT	TATTATGAGA	TAATGAACTT	GCAAAGTCAT	TATTATATAA
1634	1644	1654	1664	1674
CTTTTTTTTT	ATACTTTGAT	TTAAGAATTC	ATTTTTCTCA	TTTTATATAA
1684	1694	1704	1714	1724
ACTTATTTTT	CAACAGAAAA	TATTTTTTCGA	ACTATTCAAA	CACACCCTAA
1734	1744	1754	1764	1774
GACATTACAT	ATATATATAT	ATACACCCTC	CGTTTTATAT	TACTTAATGC
1784	1794	1804	1814	1824
CTATTGAGTT	GGCCCACCCT	TTAAGAATGA	TTCAATTAGA	GATATGTTTT
1834	1844	1854	1864	1874
ACTAAATTAA	CCTATGCTTT	AAGACTCTAA	ATTTGGCTAT	TACTATTTTA
1884	1894	1904	1914	1924
CGTTGTAATT	TAATGACAAA	CATTTCATAA	TGACTATAGT	CTGAACTTAA
1934	1944	1954	1964	1974
TTAGACAGAC	GTATCTATAG	TTTGCTTACT	AATGATTCAT	AGCTATATAT
1984	1994	2004	2014	2024
TTGGAGAGGA	GAGAGACAAA	CGATATTAAG	AAAGGGAGGA	GAGAGGCGAG
2034	2044	2054	2064	2074
GTAAATCTGA	AATAGAGAAG	AGAAAGGCAA	CCAATTTTGA	TCATCTATCA
2084	2094	2104	2114	2124
TACTTTTGAT	TATTATTTTT	ATTATATGTA	CGTTTACATT	ACAGTTTTTCG

FIG. 4-2

2134	2144	2154	2164
AATTCTTACA	TTAATCTTAA	TCATAATATA	TACA GTT GAT ATG
			Val Asp MET
2173	2182	2191	2200
TCT GGA ATT TCG AAA ATG CAA GTG ATG GCT CTT CGA GAC			
Ser Gly Ile Ser Lys MET Gln Val MET Ala Leu Arg Asp			
2209	2218	2227	2236
ATA CCC CCA CAA GAA ACA TTG CTG AAA ATG AAG CTA CTT			
Ile Pro Pro Gln Glu Thr Leu Leu Lys MET Lys Leu Leu			
2254	2263	2272	2281
CCC ACA AAT ATT TTG GGA CTT TGT AAC GAA CCT TGC AGC			
Pro Thr Asn Ile Leu Gly Leu Cys Asn Glu Pro Cys Ser			
2290	2299	2308	2317
TCA AAC TCT GAT TGC ATC GGA ATT ACC CTT TGC CAA TTT			
Ser Asn Ser Asp Cys Ile Gly Ile Thr Leu Cys Gln Phe			
2326	2335	2344	2353
TGT AAG GAG AAG ACG GAC CAG TAT GGT TTA ACA TAC CGT			
Cys Lys Glu Lys Thr Asp Gln Tyr Gly Leu Thr Tyr Arg			
2371	2380	2393	2403
ACA TGC AAC CTG TTG CCT TGA ACAATATCAA TGATCTATCG			
Thr Cys Asn Leu Leu Pro			
2413	2423	2433	2443
ATCGATCTAT CTATCTATTT ATCTGTCTCT GCGCGTATAG TGTTGTCTGT			
2463	2473	2483	2493
ACCTTTGGTG TGAAGAATAT GAATAAAGGG ATACATATAT CTAGATATAT			
2513	2523	2533	2543
TCTAGGTAAT GTCCTATTGT ATTTAAAATT TGTAGCAATG ATTGTTTGAA			
2563	2573	2583	2593
TAAAAACATA CCATGAGTGA AATAATTATT CCACATTAAT TCACGTATTT			
2613	2623	2633	2643
ATTTCACTTA TGATACGTAT TTTTGTTCCT TTCGCGTAGA TTTTGTGATCC			
2663	2673	2683	2693
TTTTCCCTTT TGAATATTAA ACATTAAACA CAAATAATGT TTATTAAATT			
2713	2723	2733	2743
AAGTTAATAT TTTTATTTAG CTATTTATAT TTTTATTTGA AATCAAACCTT			
2763	2773	2783	2793
GATAAATATT TATAAAGATA ATTAACAAGT AATGTGACAC TAACACCATG			
2813	2823	2833	2843
TAATATTATC TTGTCGTTAT TTATGATAAT ATTTTAAAAT TATAATTTCA			
2863	2873	2883	2893
GTTAAAAAAT TATTAAAAAA ACATACTTTT AAAAAGTGAG TTAGCCTCCG			
2913	2923	2933	2943
CTACCCACAT ACTTATGAAT TGGACTAGTT GTTTTTTGAC CCACAAAAAG			
2963	2973	2983	2993
AATGGGCTAA TTAAACCTGA CCTATCAAAT TTCAGAATCT GCATAGATTA			

FIG. 4-3

3013	3023	3033	3043	3053
GTCCGAACGA	AATGAGTCAG	CCCGTATTGA	ACAAAATATC	AACAAGGACG
3063	3073	3083	3093	3103
TTATGTAAAG	ATGTTTAAGA	AGGAAAAAAG	ATTTCTAATA	CATATGGACT
3113	3123	3133	3143	3153
TTCAATATCC	CAACTTTGTC	TGGCGATCTG	AACCCTGCTT	AGTTTGTGTA
3163	3173	3183	3193	3203
TCATTAACCT	GTCTTGCTAT	GTATTTAAGA	TTTAAACTTT	ATATGTTTAA
3213	3223	3233	3243	3253
ACTTACAGAA	AATACATATA	AATCTCTCAA	GACTTGGCAA	CATAATTTAC
3263	3273	3283	3293	3303
TTTAGTACTT	AAACTACATG	AAAATTTAAA	TATCCTTTTA	ACATCTTTGA
3313	3323	3333	3343	3353
AGTGAATTAA	ATTATCACAA	TCCGAGCCTA	CACCTTGGAC	GTGGCCGGCA
3363	3373	3383	3393	3403
CTCAAGAACC	AGTGCTGGTC	CCCAAGCTAA	CCCTCATCCT	GACTGACTAC
3413	3423	3433	3443	3453
AAGCGGAAGG	CTAACTTAAG	TATACAAAAG	CTTAAAACTG	AATAAAATAA
3463	3473	3483	3493	3503
ACTTTACAAG	GTTTTAACAC	AAATGAACAA	CTTTGAAGAA	AATAATATAT
3513	3523	3533	3543	3553
TCAACTAGCC	ATAAAATAGA	CAACTTTAGT	CTTTAAAACA	TTTAATAAAA
3563	3573	3583	3593	3603
TAAATGCAAA	ATATAGACTC	CTTAACTAAA	CTGACTATCT	ATGGAGCCTC
3613	3623	3633	3643	3653
TAATTGATAA	AGATGGAAGT	CGGGACAAGA	CCACGACATC	CTGACTAAAC
3663	3673	3683	3693	3703
TGAGAAGTAA	ATAAAATCCC	CCGGAAAAAA	AGGAGCCTCA	CCATGGCTAA
3713	3723	3733	3743	3753
CTCGAACTCG	GGGATATATC	AATGAAGCTC	CTGTTGATGA	TCTTGAAGAC
3763	3773	3783	3793	3803
ATGTCTCTGC	ATCATCAAAA	AGATGCAGGC	CAAATGGCTC	AGTACGTAAA
3813	3823	3833	3843	3853
ATGTACGAGT	ATGTAAGGGA	AATTCTAAAG	TATAACATAA	GCTTGATACT
3863	3873	3883	3893	3903
TGAATAAAAG	GAAACATACT	TACCTCTTTT	CAACTCAACT	CAAATTAAGA
3913	3923	3933	3943	3953
ATAAGATACT	CAACTCAAAG	ATTAGGTATT	CAACGCAAAT	ATGGCACTCT
3963	3973	3983	3993	4003
ACTCAATGAA	GTACAAATTA	ACTCAGGATA	CTCGACTTAA	GATACTCAAC
4013	4023	4033	4043	4053
TCCCGACACT	CAACTGAACT	CATTTCAATA	TAAAGCAGCT	TAAAACAAGT
4063	4073	4083	4093	4103
TCAGTATAAA	GTAAAGTTGT	TTAAAAACAT	GATGTCAACT	CTGTGTGTAT
4113	4123	4133	4143	4153
AATAAGGGAT	ACAACATAAC	TTTGAAATGT	ATATAAAAAT	ACAATTAACT

FIG. 4-4

4163	4173	4183	4193	4203
GATGTATATA	AAAATACATT	AATCTATGGG	AGATTCTCTA	ACCGACAACC
4213	4223	4233	4243	4253
ATCACTTAAG	GGCTAAGATG	ATGATATAGC	GATCTACCGC	ACGCTGCCAT
4263	4273	4283	4293	4303
CGCATCTTAT	ACCCGGCCAA	AGGTATAAGA	CCTGAACTGC	CTAATGAATC
4313	4323	4333	4343	4353
CACTAATAAA	CTGTTAAAAG	GAATCATCTA	AAAAGTATGA	CCCTTTTCTA
4363	4373	4383	4393	4403
CCCATAGTGG	CTAACATGGT	TTATGGGGGC	TGTGAGTTAT	CTGAACTCTC
4413	4423	4433	4443	4453
CCCCATATCG	GTGCTCAATA	CTACTCCAAA	AAATATACTG	CTCTTATGTT
4463	4473	4483	4493	4503
TAAAAACATA	CTGATTCTGT	GGTTTGAAAT	TATTGCTTAA	AGCTTAGATT
4513	4523	4533	4543	4553
TTTGAAAAGC	TCTCTTTTGA	AAATCGTAGT	TTCCTTTTTT	TTCTATTAAA
4563	4573	4583	4593	4603
GCTAGACATA	GGCTATGTAG	AACTCTAGCT	TACCTTCCTT	CTCAAAAGTT
4613	4623	4633	4643	4653
TGAAAACATT	TGCTTAGATT	CTTAGGGACT	ACTTAGTTCC	CTTGTTGGAA
TTC				

FIG. 4-5

PG GENOMIC

10	20	30	40	50
AAGCTTCTTA	AAAAGGCAAA	TTGATTAATT	TGAAGTCAAA	ATAATTAATT
60	70	80	90	100
ATAACAGTGG	TAAAGCACCT	TAAGAAACCA	TAGTTTGAAA	GGTTACCAAT
110	120	130	140	150
GCGCTATATA	TTAATCAACT	TGATAATATA	AAAAAAATTT	CAATTCGAAA
160	170	180	190	200
AGGGCCTAAA	ATATTCTCAA	AGTATTCGAA	ATGGTACAAA	ACTACCATCC
210	220	230	240	250
GTCCACCTAT	TGACTCCAAA	ATAAAATTAT	TATCCACCTT	TGAGTTTAAA
260	270	280	290	300
ATTGACTACT	TATATAACAA	TTCTAAATTT	AAACTATTTT	AATACTTTTA
310	320	330	340	350
AAAATACATG	GCGTTCAAAT	ATTTAATATA	ATTTAATTTA	TGAATATCAT
360	370	380	390	400
TTATAAACCA	ACCAACTACC	AACTCATTA	TCATTAAATC	CCACCCAAAT
410	420	430	440	450
TCTACTATCA	AAATTGTCCT	AAACACTACT	AAAACAAGAC	GAAATTGTTC
460	470	480	490	500
GAGTCCGAAT	CGAAGCACCA	ATCTAATTTA	GGTTGAGCCG	CATATTTAGG
510	520	530	540	550
AGGACACTTT	CAATAGTATT	TTTTTCAAGC	ATGAATTGGA	AATTTAAGAT
560	570	580	590	600
TAATGGTAAA	GAAGTAGTAC	ATCCCGAATT	AATTCATGCC	TTTTTTAAAT
610	620	630	640	650
ATAATTATAT	AAATATTTAT	GATTTGTTTT	AAATATTAAA	ACTTGAATAT
660	670	680	690	700
ATTATTTTTT	TAAAAATTAT	CTATTAAGTA	CCATCACATA	ATTGAGACGA
710	720	730	740	750
AGGAATAATT	AAGATGAACA	TAGTGTTTAA	TTAGTAATGG	ATGGGTAGTA

FIG. 5-1

760	770	780	790	800
AATTTATTTA	TAAATTATAT	CAATAAGTTA	AATTATAACA	AATATTTGAG
810	820	830	840	850
CGCCATGTAT	TTTAAAAAAT	ATTAAATAGT	TTGAATTTAA	AACCGTTAGA
860	870	880	890	900
TAAATGGTCA	ATTTTGAACC	CAAAAGTGGA	TGAGAAGGGT	ATTTTAGAGC
910	920	930	940	950
CAATAGGRGG	ATGAGAAGGA	TATTTTGAAG	CCAATATGTG	ATGGATGAAG
960	970	980	990	1000
GATAATTTTG	TATCATTTCT	AATACTTTAA	AGATATTTTA	GGTCATTTTC
1010	1020	1030	1040	1050
CCTTCTTTAG	TTTATAGACT	ATAGTGTTAG	TTCATCGAAT	ATCATCTATT
1060	1070	1080	1090	1100
ATTTCCGTCT	TAAATTATTT	TTTATTTTAT	AAATTTTTTA	AAAATAAATT
1110	1120	1130	1140	1150
ATTTTTTTCCA	TTTAACTTTG	ATTGTAATTA	ATTTTTTAAA	ATTACCAACA
1160	1170	1180	1190	1200
TATAAATAAA	ATTAATATTT	AACAAAGAAT	TGTAACATAA	TATTTTTTTTA
1210	1220	1230	1240	1250
ATTATTCAAA	ATAAATATTT	TTAAACATCA	TATAAAAGAA	ATACGACAAA
1260	1270	1280	1290	1300
AAAATTGAGA	CGGGAGAAGA	CAAGCCAGAC	AAAAATGTCC	AAGAAACTCT
1310	1320	1330	1340	1350
TTCGTCTAAA	TATCTCTCAT	CCAAACTAAT	ATAATACCCA	TTATAATTAA
1360	1370	1380	1390	1400
CCATATTGAC	CAACTCAAAC	CCCTTAAAAT	CTATAAATAG	ACAAACCCTT
1410	1420	1430	1440	1450
CCCATACCTC	TTATCATAAA	AAAAATAATA	ATCTTTTTTCA	ATAGACAAGT
1460	1470	1480	1490	1500
TTAAAAACCA	TACCATATAA	CAATATATCA	TGGTTATCCA	AAGGAATAGT

FIG. 5-2

1510	1520	1530	1540	1550
ATTCTCCTTC	TCATTATTAT	TTTTGCTTCA	TCAATTTCAA	CTTGTAGAAG
1560	1570	1580	1590	1600
CAATGTTATT	GATGACAATT	TATTCAAACA	AGTTTATGAT	AATATTCTTG
1610	1620	1630	1640	1650
AACAAGAATT	TGCTCATGAT	TTTCAAGCTT	ATCTTTCTTA	TTTGAGCAAA
1660	1670	1680	1690	1700
AATATTGAAA	GCAACAATAA	TATTGACAAG	GTTGATAAAA	ATGGGATTAA
1710	1720	1730	1740	1750
AGTGATTAAT	GTACTIONAGCT	TTGGAGCTAA	GGGTGATGGA	AAAACATATG
1760	1770	1780	1790	1800
ATAATATTGT	AAGTATTTAA	ATATTGGAAT	ATATTTGTGG	GGATGAAAAT
1810	1820	1830	1840	1850
GATAGAGAAT	ATAAGAATTA	TTTGGAAGGA	TGAAAAGTTA	TATTTTATAA
1860	1870	1880	1890	1900
AGTAGAAAAT	TATTTTCTCG	TTTTTAGTAA	TTAAAGGTGA	AAAATGAGTT
1910	1920	1930	1940	1950
TTCTCGTAAG	CGAGGAAAGT	CATTTTCCAT	GGAAGTGTAT	TTTTTTTTTA
1960	1970	1980	1990	2000
CTTTTAATAA	CGTCATAGTA	TTTGCTATAC	TCAAGAATAA	GACACTATTA
2010	2020	2030	2040	2050
TTGATGTTTA	GTGCTCGAAA	AGAAATTGAT	AGTAATTTTG	CTAATATAAC
2060	2070	2080	2090	2100
TATCAATTTT	TTATATGTAT	ATTTTTC AAC	CAAAATAACA	AAGCGTAATC
2110	2120	2130	2140	2150
CAATAAGTGG	GCCTCTAGAA	TAAAGAGTAA	GTTCTATTAA	TTCTTAACCT
2160	2170	2180	2190	2200
TATTTAATTT	TATGGAAACC	TCGACAAAAC	GACAATGCTC	AACTTATATT

CGAATTC

FIG. 5-3

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US88/01811**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (4): C07H 15/12 C12N 15/00 C12N 5/00 A01H 1/04
U.S. CL: 536/27 435/172.3 435/320 435/240.4 800/1

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
U.S.	435/172.3, 240.4, 320 536/27 800/1

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages †	Relevant to Claim No. ‡
X Y	Plant Physiology, Volume 83, issued April 1987, (Rockville, Maryland, USA), Boston et al., "Expression from heterologous promoters in electroporated carrot protoplasts", pages 742-746, see pages 742-743 in particular.	1-3, 6, 9, 12, 13, 16, 17 8, 14, 18-24
Y	Molecular and General Genetics, Volume 200, issued August 1985, (Heidelberg, Germany), Mansson et al., "Characterization of fruit specific cDNAs from tomato", pages 356-361, see pages 356, 358 and 360 in particular.	1-6, 10, 14, 15, 18-24

* Special categories of cited documents: †‡

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

23 JULY 1988

Date of Mailing of this International Search Report

07 SEP 1988

International Searching Authority

ISA/US

Signature of Authorized Officer

DAVID T. FOX

David T. Fox

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>Bio/Technology</u> , Volume 3, issued March 1985, (New York, New York, USA), Facciotti et al., "Light-inducible expression of a chimeric gene in soybean tissue transformed with Agrobacterium," pages 241-246, see page 241 in particular.	1-3,8,9, 11,14, 18-24
Y,P	<u>Molecular and General Genetics</u> , Volume 208, issued June 1987, (Heidelberg, Germany), Sheehy et al., "Molecular characterization of tomato fruit polygalacturonase", pages 30-36, see pages 30 and 33 in particular.	7,14,18, 23
Y	<u>Proceedings of the National Academy of Sciences USA</u> , Volume 83, issued September 1986, (Washington, D.C., USA), Della Penna et al., "Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening," pages 6420-6424, see page 6422 in particular.	7,14,18, 23
Y	<u>Nucleic Acids Research</u> , Volume 14, issued November 1986, (Oxford, England), Grierson et al., "Sequencing and identification of a cDNA clone for tomato polygalacturonase," pages 8595-8603, see pages 8598-8599 in particular.	7,14,18, 23
Y	<u>Proceedings of the National Academy of Sciences USA</u> , Volume 83, issued August 1986, (Washington, D.C., USA), Ecker et al., "Inhibition of gene expression in plant cells by expression of antisense RNA," pages 5372-5376, see page 5373 in particular.	5,7,14, 23

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

Proceedings of the National Academy of Sciences USA, Volume 82,
issued May 1985, (Washington, D.C., U.S.A.), Sengupta-Gopalan et al., "Developmentally regulated expression of the bean beta-phaseolin gene in tobacco seed," pages 3320-3324, see page 3321 in particular.

1-3,8,9,
11,14,
18-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:
3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.